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AWARD NUMBER DAMD17-96-1-6223

TITLE: Training in support of research entitled "Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer".

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REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE
Training in support of research entitled "Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer".

5. FUNDING NUMBERS
DAMD17-96-1-6223

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 words)***

Overexpression and/or amplification of *HER-2/neu* has been implicated in the genesis of a number of human cancers, especially breast and ovarian cancers. Transcriptional upregulation has been shown to contribute significantly to the overexpression of this gene. PEA3 is a DNA binding transcriptional factor and its consensus sequence exists on the *HER-2/neu* promoter. We transfected PEA3 into the human breast and ovarian cancer cells that overexpress *HER-2/neu* and showed that PEA3 dramatically represses *HER-2/neu* transcription. and suppresses the oncogenic *neu*-mediated transformation in mouse fibroblasts. Expression of PEA3 selectively blocks the growth of human cancer cells that overexpress *HER-2/neu*. Studies in the orthotopic ovarian cancer model demonstrated that expression of PEA3 preferentially inhibits growth and tumor development of human cancer cells that overexpress *HER-2/neu*, through down regulation of *HER-2/neu*-p185. Thus, PEA3 is a negative regulator of *HER-2/neu* gene expression and functions as a tumor suppressor gene in the *HER-2/neu*-overexpressing human cancer cells. Our studies also indicated that in the *HER-2/neu*-overexpressing cells, PEA3 competes with a transactivator for binding to the PEA3 site, preventing the putative factor from activating the transcription of *HER-2/neu*.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Breast Cancer	PEA3, Tumor suppression, HER-2/neu, Transcriptional repression	45
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Amplification and overexpression of the *HER-2/neu* gene were first found in human breast cancers and later in ovarian cancers (1-4). It was also reported that the overall survival rate and time until relapse for breast and ovarian cancer patients having *HER-2/neu* overexpression is significantly shorter than that for patients not having *HER-2/neu* overexpression (4, 5). In addition to breast and ovarian cancers, *HER-2/neu* overexpression has recently been found in lung (6, 7), gastroinstestinal tract (8) and oral cancers (9) with high frequency, suggesting that *HER-2/neu* overexpression plays a critical role in the development of human cell malignancy. Interestingly, *HER-2/neu* overexpression was also found to cause enhanced metastatic potential (10, 11) and to induce chemoresistance (12, 13) in human cancer cells. *HER-2/neu* may significantly contribute to the poor clinical outcome of patients with *HER-2/neu*-overexpressing cancers, and it is an excellent target for investigating the mechanism of cellular transformation and for seeking new therapeutic strategies.

Transcriptional upregulation has been shown to contribute to *HER-2/neu* overexpression in different breast cancer cells (14, 15). We previously showed that the adenovirus E1A effectively represses *HER-2/neu* transcription and inhibits malignant transformation of *HER-2/neu*-overexpressing cancer cells (16-18), indicating that repression of transcription may be an effective way to reverse the malignant transformation mediated by *HER-2/neu* overexpression. PEA3 is an *ets*-family transcriptional factor that binds to the promoter region at the PEA3 site and regulates the expression of many genes involved in cell growth and differentiation (19). We identified that the PEA3 binding site (AGGAAG) resides on the *HER-2/neu* promoter. We were interested in studying the role of PEA3 in *HER-2/neu* gene expression and *HER-2/neu*-mediated transformation.

In the literature, many PEA3 target genes have been reported. A significant fraction of these genes encode proteinases that are required for degradation of the extracellular matrix. For example, PEA3 directly activates transcription from the MMP-1, MMP-9 and MMP-11 promoters (20, 21). In agreement with these findings, forced expression of PEA3 in the non-metastatic MCF-7 human breast cancer cell line increases its invasive and metastatic properties (22). Hence PEA3 may be a regulator of the invasive and metastatic phenotype. The metastatic suppressor gene maspin is characterized as a tumor suppresser gene, it expressed low in human cancer cells than in normal cells. The PEA3 and Ap1 play a positive role in the transcriptional regulation of this gene and the loss of maspin expression is at least in part due to the lack of PEA3 and Ap1 transactivation activity(23). Therefore, in this scenario, the expression of PEA3 helps to prevent cell transformation.

A series of 20 breast-cancer cell lines and 2 normal breast epithelial cell lines were tested for their expression of PEA3 gene by northern blot analysis (24). There was no detectable PEA3 mRNA in the *HER-2/neu*-overexpressing MDA MB134-V, MDA MB453 and ZR-75-1 cells. The PEA3 mRNA level was low in the other *HER-2/neu*-overexpresser such as BT474 and SKBR-3 and MDA MB361. The recent demonstration that *Ets*-responsive elements are contained within a number of genes implicated in the development, progression and invasion of breast cancer, e.g., the matrix metalloproteinase enzymes and the *c-erbB*-2 oncogene, also argues that these molecules may play an important role in breast cancer.

Overexpression of the PEA3 gene occurs in mammary tumors of transgenic mice engineered to express the rat *neu* gene in their mammary epithelium (25). In this experimental system *HER-2/neu* induces metastatic mammary tumors thereby closely mimicking the human disease (26). All primary and metastatic mammary tumors of

these transgenic mice overexpress PEA3 mRNA, whereas normal tissues adjacent to the primary tumor or metastases do not (25). These correlations indicate that PEA3 plays certain role in the mammary tumorigenesis. However, how PEA3 involves in the development of mammary tumor, and how the expression of *HER-2/neu* affects the PEA3 gene or vice versa need to be further studied.

Our experimental data demonstrate that PEA3 represses the *HER-2/neu* transcription. Thus, we hypothesized that PEA3 may function as a tumor suppressor for *HER-2/neu*-overexpressing breast cancer cells and sensitize response of breast cancer cells to chemotherapeutic drugs through PEA3-mediated *HER-2/neu* repression. Our study has been primarily focused on investigating the effects of PEA3 expression on *HER-2/neu* transcription, *HER-2/neu*-mediated transformation, *HER-2/neu*-overexpressing human cancer cell growth and tumorigenesis. Our data demonstrate that PEA3 represses *HER-2/neu* transcription in human cancer cells and suppresses *neu*-mediated transformation in mouse-fibroblast NIH3T3 cells. Reintroduction of PEA3 suppresses the growth of cancer cells that overexpress *HER-2/neu,* but not those with a basal level of *HER-2/neu* expression. Site-directed mutagenesis indicates that PEA3-induced trans-repression of the *HER-2/neu* promoter might involve competition between PEA3 and another *ets*-related transcriptional activator, which contributes to the transformating phenotype of *HER-2/neu.* The DNA binding domain of PEA3 is sufficient for the transcriptional repression of *HER-2/neu.*

Body

Experimental methods and procedures

Plasmids. For the *HER-2/neu* promoter-driven luciferase-expressing vector, the promoter region of human *HER-2/neu* was amplified with a pair of primers (sense: 5'-GATAGGATCCGGGGGTCCTGGAAGCC; antisense: 5'-GGGCAGATCTGGTTTTCCGGTCCCAATGGA). The amplified DNA fragment was treated with BamHI and BglII, and ligated into the BamHI site of pGL2-Basic (Promega, Madison, WI); the resultant plasmid was named pNeulit. The sequence of this insert was confirmed.

For site-directed mutagenesis construction of pm-pNeulit, two pairs of primers were used for overlapping PCR, including the primers mentioned above and the other pair of primers spanning the PEA3 site on the *HER-2/neu* promoter (PEA3 motif AGGAAG was changed to ACGAGC) (antisense: 5'-CATTCTTATACGAGCTCAAGCTCCTCC; sense: 5'-GGAGGAGGAGGGCTGCTTGAGCTCGTATAAGAATG). The PCR fragment was digested with BamHI and BglII, ligated and sequenced as for pNeulit.

The full-length PEA3 cDNA was cloned into the ClaI/BamHI site of the vector pSRα to make pSRaPEA3 and PEA3 CDNA was cut out from pSRaPEA3 by HindIII and BamHI and cloned into the same sites of pCDNA3 vector to form pCDNA3-PEA3.

Cells. Mouse fibroblast NIH3T3 and human cancer cell MDA MB453, MDA MB435, SK-BR-3, SKOV3.ip1 and 2774 c-10 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and 10% fetal bovine serum, respectively.

Focus forming assay. NIH3T3 cells ($2x10^5$) were plated to a 100-mm culture dish 24 hours before transfection. Cells were transfected with 0.5 μg of pSV2neo, 1 μg of cNu104 plus 5 μg of pSRαPEA3 or pSV2E, or 0.5 μg of pSV2neo plus 6 μg of

pSRαPEA3 by a modified calcium phosphate precipitation technique. Two days after transfection, cells were distributed to 4 plates; 2 were selected in 500 μg/ml of G418 (Life Technologies, Inc), and the other two maintained in normal medium. Media were changed every 3 days until foci appeared. The number of foci was normalized by number of neomycin-resistant colonies.

Gel mobility shift assay. The oligonucleotide derived from *HER-2/neu* promoter (with wild-type PEA3 site: 5'- GGAGCTCGAGGGCTGCTTGAGGAAGTATAAGAATG-3'; with PEA3 site mutated: GGAGCTCGAGGGCTGCTTGAGGTCGTATAAGAATG-3') was end labeled by [^{32}P]γATP. Binding of the protein factors to DNA sequences was achieved in a mixture containing 1X binding buffer (20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 5% glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT), 1 μg GST-PEA3 (GST or MB453 nuclear extract), 2-8 μg poly d(I-C), 1 μg of bovine serum albumin, or 100X the amount of competitor oligonucliotide and 20,000 cpm of ^{32}P-labeled oligonucleotide (In the competition assay, 1-2 μg of PEA3 protein was also included in the reaction mixture). The binding reaction was performed at room temperature for 20 minutes. The samples were electrophoresed in 1X TBE in a 5% polyacrymide gel that had been pre-run at 100 V for at least 30 minutes. Gels were dried for 45 minutes under vacuum and autoradiographed.

Transient transfection and luciferase assay. The human cancer cells was seeded to 60-80% of confluency in the 100-mm petri dishes, 60-mm petri dishes or 6-well cell culture plates, and were transfected by incubating with DNA in the indicated amount and DC-chol liposome complex (in a ratio of 13 nmol lipid/1 μg of DNA) in a serum free condition for 2-5 hours, or by incubating with the DNA-polyethilenimine(PEI) complex (of 0.5 μl/μg DNA) for 1-3 hours. Cells were harvested 48 hours later, using 800 μl/100-mm dish (or 300 μl/60-mm dish or 130 μl/6-well) of 1X reporter lysis buffer (Promega). 100 μl of lysates mixed with 100 μl of assay substrate was measured the

luciferase activity using the monolight 2010 Luminometer (Analytical Luminescence Laboratory). To normalize for variations in transfection efficiency, the values obtained from luciferase values were divided by ß-galactosidase values. The ß-galactosidase assays were performed by mixing equal amounts of cell extract, and 2 X Buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM ß-mercaptoethanol, 1.33 mg/ml orthonitrophenyl galactose), incubating at 37° until a faint yellow color developed and reading the absorbance at 420 nm.

Stable transfection and colony formation assay. Different human cancer cells in 80% confluency were transfected with 1 µg of PEA3 expression plasmid or its backbone plasmid pCDNA3, using DC-Chol liposome as a carrier, and put under selection of G418 (500µg/ml - 800 µg/ml) 48 hours later. Media were changed every 3-4 days until the colonies formed. 1% crystal violet was used to stain the colonies. Transfections in each cell line were repeated 2-3 times using different passage cells and with DNA from different extractions.

RT-PCR for gene expression. The colonies derived from cancer cells in which PEA3 or backbone plasmids has been transfected were developed into cell lines; total RNA was extracted and RT-PCR was performed using the SuperScript preamplification system (Life Technologies, Inc). The primers for PEA3 PCR were: sense (from the PEA3 coding sequence) 5'-TGAATTATGACAAGCTGAGCCG; antisense (from the cDNA sequence derived from the expression vector pCDNA3): 5'-TCAGCGAGCTCTAGCATTTAGG. As the RT-PCR internal control, the primer for the GAPDH PCR were: sense 5'AGGTGAAGGTCGGAGTCAAC; antisense: 5'-TCCATTGATGACAAGCTTCCC) were used as RT-PCR control. Cos1 cells transiently transfected by the PEA3 gene were used as a positive control. Amplification was performed on a Perkin Elmer DNA Cycler 480, in a total volume of 50 µl containing 2 µl of cDNA (or 10 ng of plasmid pPEA3 as positive control, or water as the negative control) in PCR buffer (20 mM Tris-HCl, pH 8.4, 25 mM KCl, 1.5 mM

$MgCl_2$), 50 mM each dNTP, 10 pmol of each primer and 1 U of Taq polymerase (Promega) for 35 cycles of reaction with denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes and extension the primer at 72°C for 1.5 minutes.

Orthotopic human ovarian cancer nude mouse model and Liposome-mediated in vivo gene transfer. Four- to six-week-old athymic female homozygous *nu/nu* mice were purchased from Harlan Sprague Dawley, Inc.(Indianapolis, IN) and cared for and used in accordance with institutional guidelines. Mice were housed for 1 to 2 weeks, and each mouse that was considered healthy was aseptically injected i.p. 2 X 10^6 SKOV-3 cells. Five days later, the mice that had tumors were placed in five groups. The mice received weekly i.p. injections of 200 μl of different reagents for the certain period of time. The responses and survival rates were observed for 1 year. After mice dead, necropsies were performed, and tumor tissues and major organs (brain, lung, liver, heart, kidney, and spleen) were collected for further analysis.

Immunohistochemical staining. Histological sections of tumors and organs of tumor-bearing mice were fixed with formalin, embedded in paraffin, and subjected to routine pathological analysis by hematoxylin and eosin staining. The p185 protein was detected by using polyclonal antibody (DAKO corporation) as the primary antibody, and biotinylated goat anti-rabbit IgG as second antibody, followed by incubation with streptavidin-alkaline phosphatase and then development using a phosphatase substrate kit; 1% methyl green was used as a counterstain. To detect K1 protein, monoclonal antibody against PEA3 (MP16) (Santa Cruz) and biotinylated anti-mouse IgG was used, followed by incubation with ABC reagent (Vector laboratory) and then developed in ABC chromogen substrate solution; mayer's hematoxylin was used as a counterstain.

Immunoblotting. Immunoblot analysis of tumor protein lysates was performed as described previously. Protein lysates (50 μg) from tumor tissues of mice that had died were separated by 8% SDS-PAGE and immunoblotting was performed with antibody against p185 c-neu Ab-3 (Pharmingen).

Assumptions and Goals

HER-2/neu is consistently amplified and/or overexpressed in a subset of human breast cancers. Increased levels have been implicated to facilitate the tumorigenic process and the downregulation of this gene expression reverses the cell transformation. Thus, identifying a transcriptional repressor to effectively block the expression of this gene is critical to the understanding of breast cell tumorigenesis, and may lead to the cancer therapeutics. The goal of this project is to examine the effects of transcriptional factor PEA3 on *HER-2/neu* promoter activity, and focus on examining the effects of PEA3 expression on cells transformed by *HER-2/neu* overexpression. Once the suppression activities of PEA3 gene are defined, this strategy will be extrapolated to the application of other oncogenes whose overexpression induces transformation.

The specific questions to be addressed in this work are as follows:

1, Whether the PEA3 protein binds specifically to the PEA3 consensus sequence identified on the *HER-2/neu* promoter?

2. What effects PEA3 may have on the transcriptional regulation of *HER-2/neu* promoter?

3. If PEA3 has repressive activity on the *HER-2/neu* gene transcription, does it suppress the transformation mediated by *HER-2/neu* gene? If PEA3 promotes the transcription of *HER-2/neu*, is it the factor that contributes to the transcriptional upregulation of *HER-2/neu* and consequently causes the transformation in human cancer cells?

4. If PEA3 does repress transcription of the *HER-2/neu* gene, is this due to its interaction with the basal transcription factors or due to the disrupting the function of other enhancer(s) or transcriptional activator(s)?

5. If PEA3 is overexpressed in the *HER-2/neu*-overexpressing human cancer cells, How does PEA3 affect the biological property of these cells?

6. Using the orthotopic cancer model, determining the effects of PEA3 expression on the *HER-2/neu*-overexpressing cancer cell growth *in vivo*. If there were any effects, does PEA3 mediate these effects through transcriptional regulation of *HER-2/neu*-p185?

7. Using both different gene delivery systems, liposome and Adenovirus vector to test the tumor growth suppression activity mediated by PEA3 in the orthotopic breast cancer models (unfinished).

Results

PEA3 binds directly to the HER-2/neu promoter To identify transcriptional factors that inhibit *HER-2/neu* expression through the promoter, we searched for the transcriptional factor binding consensus sequences on the *HER-2/neu* promoter, and identified a PEA3-binding motif 5' AGGAAG 3' close to the transcription initiation sites of *HER-2/neu*. To examine whether PEA3 binds to the *HER-2/neu* promoter through this PEA3 motif, a gel shift analysis was performed. The bacterially expressed GST-PEA3 protein was used to interact with an oligonucleotide containing the PEA3 binding site (5'-AGGAAG-3'). A specific complex of PEA3-DNA was reproducibly detected. Formation of this complex was blocked by a 100-fold concentration of unlabeled oligonucleotide, but not by 100-fold of a mutant oligonucleotide (PEA3 binding site changed to 5'-AGCTCG-3') (Fig. 1A), suggesting that PEA3 binding to the promoter was PEA3 motif specific. To further confirm this specificity, the mutated oligonucleotide probe was incubated with the PEA3 protein; no PEA3-DNA complex was detected (Fig. 1B). These results clearly demonstrate that the PEA3 protein specifically bound to the *HER-2/neu* promoter at the PEA3 consensus site *in vitro*.

PEA3 represses HER-2/neu transcription in breast and ovarian cancer cells that overexpress HER-2/neu The effects of PEA3 on the transcription of the *HER-2/neu* gene was tested using a luciferase reporter plasmid (pNeulit), in which the luciferase gene expression is controlled by the 530 base pair from the *HER-2/neu* promoter. When pNeulit was cotransfected into SKOV-3, a *HER-2/neu*-overexpressing ovarian cancer cell line, with the effector gene PEA3 to express the PEA3 protein, the level of luciferase activity was lower compared with that of the transfection with the control effector plasmid expressing no protein. PEA3 exhibited dose-dependent repressive activities (Fig. 2A). Similar transcriptional repression was also observed in the *HER-2/neu*-

overexpressing human breast cancer cell line MDA MB453 by the cotransfection assay, using CAT as a reporter (Figure 2B). Thus, PEA3 dramatically repressed the transcriptional activity of the human *HER-2/neu* promoter.

PEA3 suppresses the transforming activity of the rat genomic activated neu To further demonstrate that the repression of *HER-2/neu* transcription by PEA3 suppresses the transformation caused by *HER-2/neu*, a focus-forming assay was performed in mouse fibroblast NIH 3T3 cells. cNu104 is a cosmid containing mutation-activated genomic rat *neu* under its own promoter. Transfection of cNu104 transformed NIH 3T3 cells, and foci formed on the top of the monolayer cells. The focus-forming ability of genomic *neu* was dramatically lower when PEA3 expression plasmids were cotransfected with cNu104; only a few foci were formed (Fig. 3). This result supports the concept that the transcriptional repressor PEA3 gene suppresses the transformation of activated *neu*.

Binding to the neu promoter is required for PEA3-mediated transcriptional repression
To examine further whether the PEA3 consensus site is required for transcriptional repression of *HER-2/neu*, a mutation on the *HER-2/neu* promoter-driven luciferase reporter gene was created by site-directed mutagenesis in which the PEA3 consensus sequence AGGAAG was changed to AGCTCG (the construct is called pm-pNeulit). At all doses tested, PEA3 failed to repress pm-pNeulit activity in MDA MB453 breast cancer cells (Figure 4A) and SKOV3.ip1 ovarian cancer cells(Figure 4B), further demonstrating that the PEA3 motif is required for PEA3-mediated transcriptional repression. We also observed that the transcriptional activity of pm-pNeulit was significantly lower than that of the wild-type *HER-2/neu* promoter construct pNeulit(Figiure 4A, B), and the only difference between those two was the PEA3 binding site. One of the interpretations is that the PEA3 site was originally an enhancer-like element in *HER-2/neu*-overexpressing cancer cells, the abolishment of this site causes

decreased transcriptional activity. We therefore hypothesized that in the *HER-2/neu*-overexpressing cells, expression of PEA3 competes for PEA3 motif with a transcriptional activating factor on the *HER-2/neu* promoter and this factor is therefore unable to activate the transcriptional activity of the *HER-2/neu* promoter.

PEA3 competes for the HER-2/neu promoter binding with a transcriptional factor To demonstrate the competition between PEA3 and the other factor for DNA binding, a gel shift assay was performed using the nuclear extract from the MDA MB453 cells, in which there is no PEA3 expression detected. The probe used is the same as the one in the Fig. 1A, an oligonucleotide derived from the *HER-2/neu* promoter spanning the PEA3 motif. We detected a retarded band representing a complex of DNA and protein factor(s) in the nuclear extract and that was specific for the PEA3 consensus sequence. The addition of purified PEA3 protein to the reaction mixture resulted in a dramatically reduction of this complex and the appearance of another band of PEA3-DNA complex in a dose-dependent manner (Figure 5). It suggests that the competitions for binding to the PEA3 site occur while PEA3 is expressed in the *HER-2/neu*-overexpressing cancer cells.

Binding to the PEA3 motif on the HER-2/neu promoter is sufficient for PEA3-mediated transcriptional repression PEA3 as a transcriptional regulator contains both transactivating domain (n-terminal portion) and DNA binding domain (c-terminal portion). The DNA binding domain of PEA3 might be sufficient for *HER-2/neu* repression based the results above. To further confirm this concept, an expression vector PEA3-DBD was constructed which encodes only the DNA binding domain of the PEA3 gene. The deletion mutant PEA3-DBD sufficiently represses the *HER-2/neu* promoter activity (Figure 6). The transcriptional regulation domain of PEA3 may not be required in the PEA3-mediated *HER-2/neu* transrepression in this system. Since the *ets*-family transcription factors share similar DNA-binding sites (such as PEA3 motif) through their

highly homologous *ets* DNA-binding domain. The factor that competes with PEA3 in our gel shift assay might be an *ets* family transcriptional factor. These data highly support the hypothesis that PEA3 binding site in the *HER-2/neu* promoter actually functions as an enhancer-like element in the *HER-2/neu*-overexpressing cancer cells to which an ets activator binds, but not the PEA3. Expression of PEA3 provides the DNA binding activity to compete with this factor, keeps it away from regulating the transcription, the *HER-2/neu* promoter activity is consequently repressed.

PEA3 expression preferentially suppresses the growth of human cancer cells that overexpress HER-2/neu To examine the biological effects of PEA3 expression on *HER-2/neu*-overexpressing human cancer cells, we intended to establish cell lines expressing PEA3 by transfection of the PEA3 expression vector containing a neomycin-resistant gene or the backbone plasmid pCDNA3 into the *HER-2/neu*-overexpressing human breast cancer cells (MDA MB453 and SK-BR-3) and ovarian cancer cells (SKOV-3.ip1). The transfected cells were subjected to G418 selection for 2-4 weeks and the drug-resistant colonies were stained with crystal violet. The transfection with the PEA3 expression plasmids consistently produced very few colonies in all these cells. It reminds us of the observation that the tumor suppressor gene p53 and pRB expression is not compatible with continued growth of cancer cells (27, 28). The colony formation of PEA3 suggests that PEA3 expression in these cells blocks the cancer cell growth and may also associate with tumor suppression activity. To examine whether PEA3 might mediate these effects through *HER-2/neu*, the same transfections were also performed in other cells expressing a basal level of the *HER-2/neu* gene (breast cancer cell MDA MB435 and ovarian cancer cell 2774 c-10). Transfection of PEA3 and its backbone plasmid generated similar numbers of drug-resistant colonies in these two cell lines. Together, these experiments strongly suggest that the expression of PEA3 can selectively suppress the growth of *HER-2/neu*-overexpressing human cancer cells (Figure 7 & Table 1). This conclusion was

further supported by the detection of PEA3 expression in the colonies. Ten colonies from PEA3 transfecting MDA MB435 cells and ten from MDA MB453 cells were examined for their PEA3 expression in RT-PCR, using a pair of PEA3 transgene specific primer. PEA3 RNA was expressed in all PEA3-transfected colonies from MDA MB435 while only expressed in 2 of 10 colonies obtained from *HER-2/neu*-overexpressing MDA MB453 cells (Figure 8). These data strongly support that PEA3 expression preferentially suppresses the growth of *HER-2/neu*-overexpressing cells. This phenomenon does not seem to occur in the cells that express basal level of *HER-2/neu*.

PEA3 preferentially inhibits the growth and the tumor development of cancer cells that overexpress neu *HER-2/neu* overexpression in human cancer cells has been implicated in the genesis of a number of human tumors. The experimental data shown in Figure 7 indirectly showed the blockage of *HER-2/neu*-overexpressing human cancer cell growth by the PEA3 gene. These observation prompted us to demonstrate whether *in vivo* PEA3 expression may suppress cell growth, by inducing the *HER-2/neu*-overexpressing ovarian cancer in the preclinical animal model and using the cationic liposome to deliver PEA3 to those cancer cells.

Tumors were induced in nude (nu/nu) mice by injecting i.p. SKOV3.ip1 cells in which *HER-2/neu* is overexpressed. Five days later, we started weekly i.p. injections of 200 μl of PEA3-liposome complex or proper controls. The mice from control groups died of tumors and ascites within 6 months. However, the mice that had received PEA3-liposome complex treatment survived longer than did the controls, and 50% of the mice were alive and healthy without palpable tumors after 12 months. Immunoblot analysis demonstrated that the tumor samples from the PEA3-liposome-treated mice had lower levels of p185 expression than did samples from control mice (Figure 9). To further demonstrate the correlation between the growth inhibition effects of PEA3 expression and *HER-2/neu* gene repression, immunohistochemical staining was performed on tumor

tissues obtained from mice on the day they died. In one of the SKOV3.ip1 tumors derived from mice treated with the complex, PEA3 protein staining was positive in approximately 30% of cancer cells, whereas the*HER-2/neu*-encoded p185 protein staining was negative in approximately 50% of cells. Cancer cells from PBS-treated mice showed 100% positive staining for p185. Thus, downregulation of p185 expression might be the mechanism of PEA3-mediated inhibition of cell growth and tumor development. To test this possibility, we used another human ovarian cancer cell line, 2774 c-10, which expresses a basal level of *HER-2/neu,* to establish tumors in nude mice with the same procedure. In the 2774 c-10 groups, mice treated with either PEA3-liposome or PBS died of malignant tumors and ascites within 5 months (Figure 10). Tissue sections derived from the 2774 c-10 tumors treated with PEA3 were analyzed by immunohistochemical staining. PEA3 protein was expressed in approximately 40% of the tumor cells. Thus, PEA3 suppressed tumor cell growth preferentially in *HER-2/neu*-overexpressing cancer cells, most likely through targeting of *HER-2/neu* gene expression.

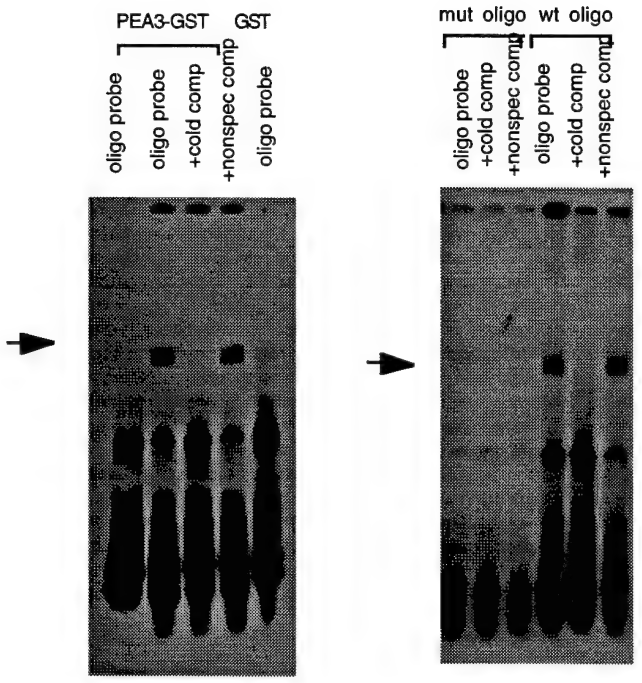


Figure 1 *Binding of PEA3 to HER-2/neu promoter in vitro.* ***A****, Radiolabeled oligonucleotide probe derived from HER-2/neu promoter spanning the PEA3 motif was end-labeled using [^{32}p]-γATP. 1 μg of GST-PEA3 fusion protein was used to interact with the oligonucleotide alone or in the presence of 100-fold of the same cold oligonucleotide, or in the presence of 100-fold of cold oligonucleotide with mutation in the PEA3 binding site. As a control, 1 μg of GST protein was also incubated with the labeled probe.* ***B****, The same as A, except that the PEA3 protein was used to interact with both the oligonucleotide probe from HER-2/neu promoter, and the oligonucleotide probe with mutation at PEA3 motif.*

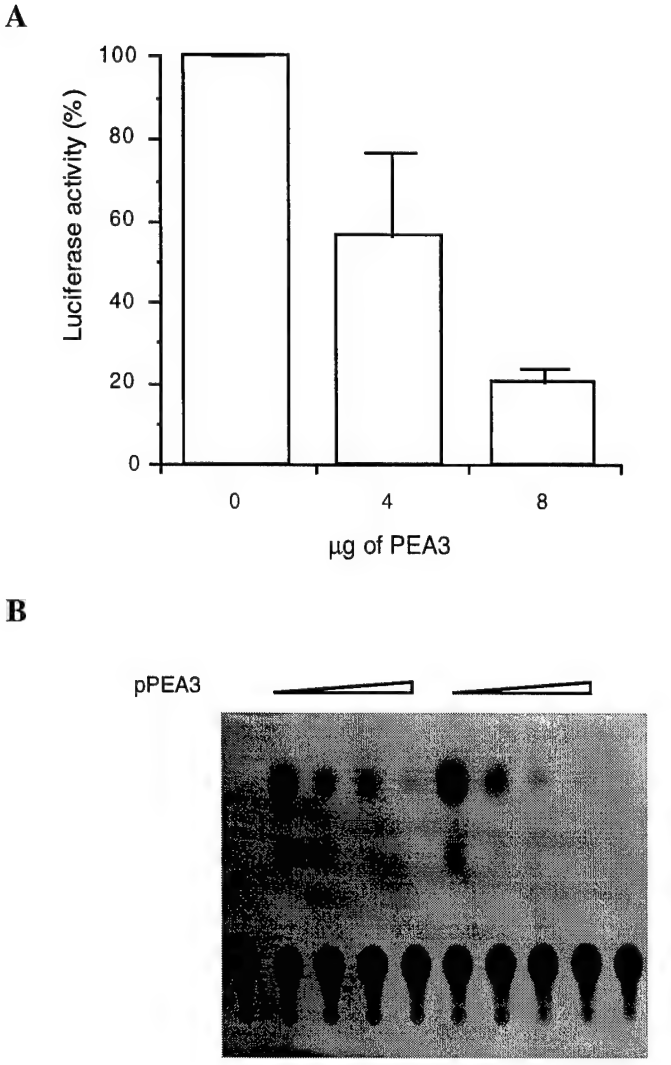


Figure 2 *PEA3 inhibits HER-2/neu promoter activity in human breast and ovarian cancer cells. PEA3 exhibits dose-dependent repressive activity when cotransfected with the HER-2/neu promoter-driven luciferase reporter in the HER-2/neu-overexpressing ovarian cancer cell line SKOV-3* ***(A)*** *or with the CAT reporter in the HER-2/neu-overexpressing human breast cancer cell line MDA MB453* ***(B).*** *The doses of PEA3 in the transfections are 0, 5, 10, and 15 µg in Panel B.*

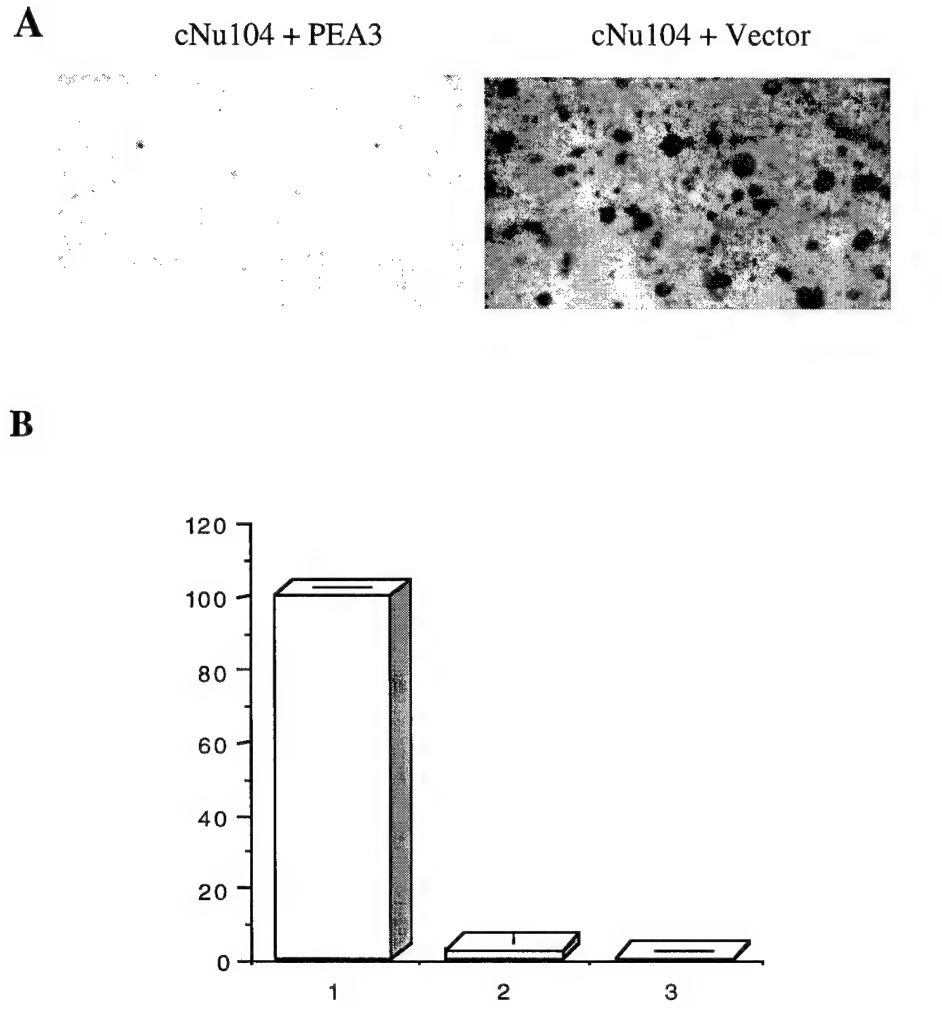


Figure 3 *PEA3 suppresses neu-mediated transformation. Rat genomic activated neu (cNu104) was transfected into NIH3T3 cells to form foci. The PEA3 was cotransfected with cNu104 to test its ability to suppress the transformation. pSV2-neo was included in each transfection as the internal control* ***(A).*** *The focus formation was expressed as the ratio of foci to colonies from each transfection to normalized transfection efficiency, and the focus formation of cNu104 transfection was set as 100%* ***(B).***

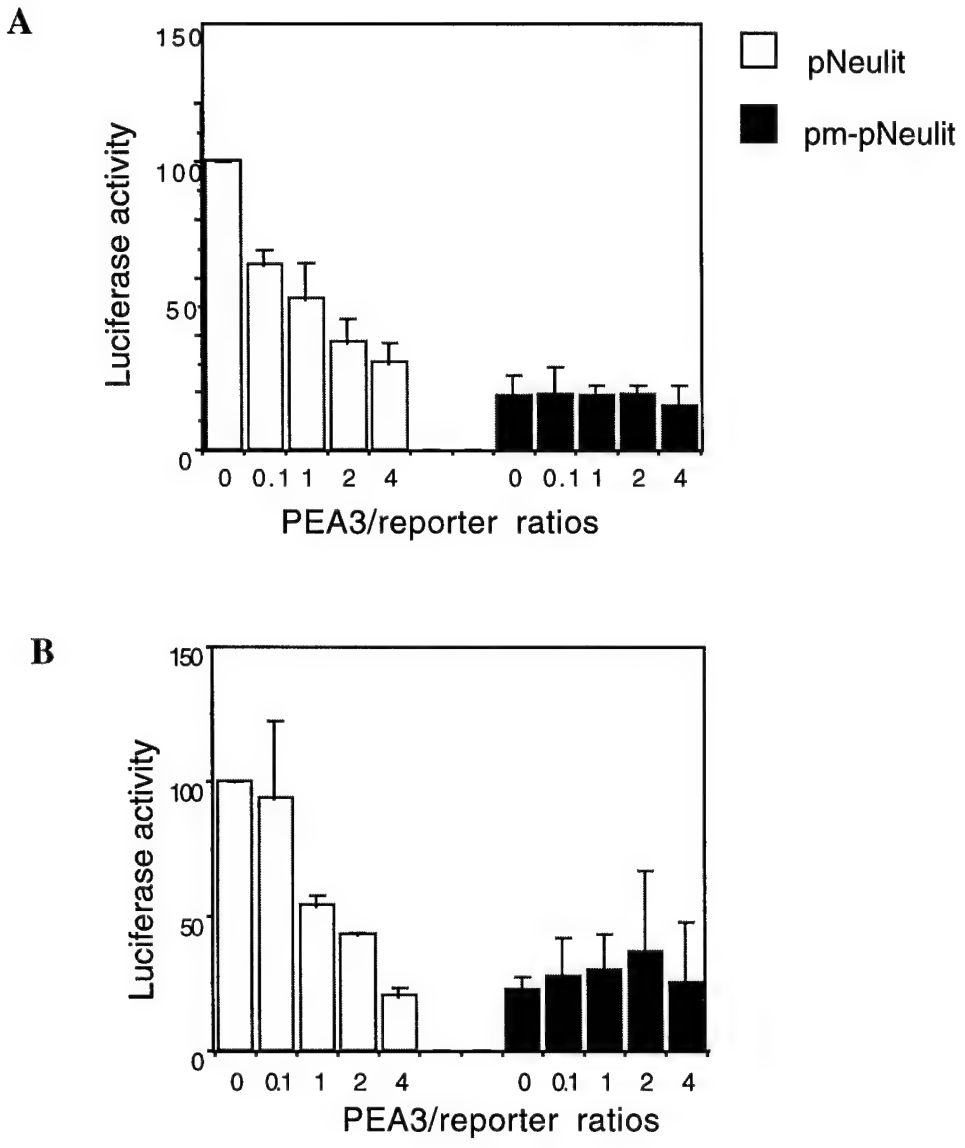


Figure 4 *PEA3 regulates HER-2/neu promoter through ets consensus site. Human breast cancer cells MDA MB453* ***(A)*** *and ovarian cancer cells SKOV-3* ***(B)*** *were cotransfected with PEA3 plasmid and HER-2/neu promoter luciferase reporter pNeulit or its counterpart that mutates the ets site pm-pNeulit. Bar, SD. The activity of pNeulit was set to 100%*

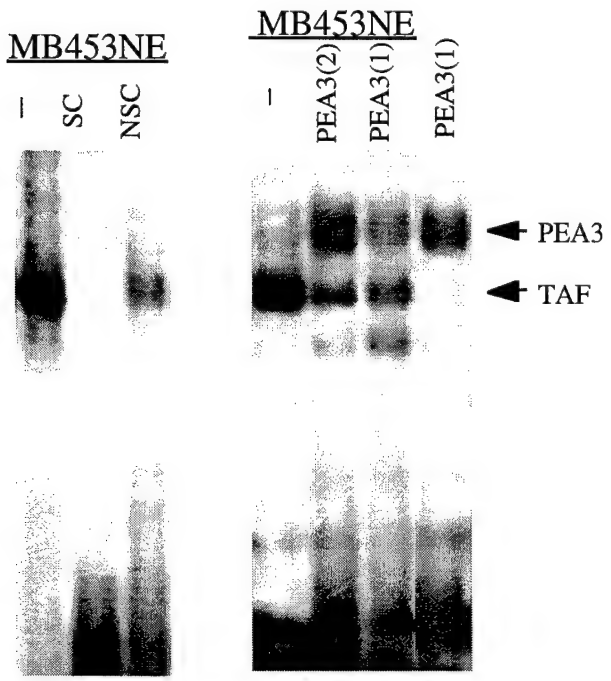


Figure 5 *PEA3 competes with another factor for binding to HER-2/neu promoter. Same gel shift assay as in Figure 3.1.1A except that 1 μg of nuclear extract from MDA MB453 cells was used to complex with the probe and the PEA3 protein of 2 or 1 μl (i.e. 0.6 or 0.3 μg) was included for competition.*

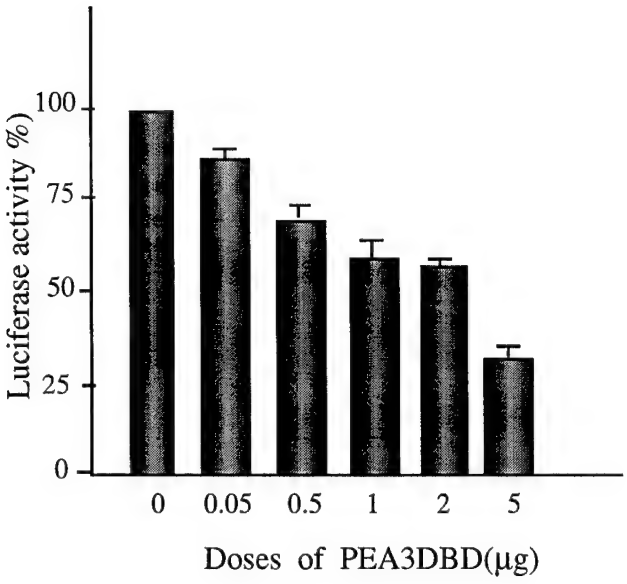
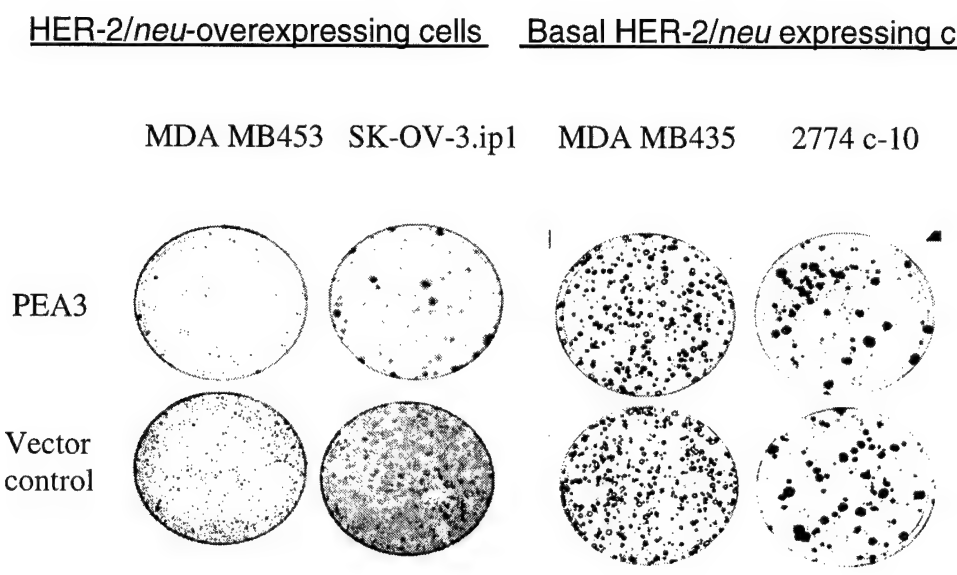


Figure 6 *DNA Binding domain of PEA3 protein is sufficient for the transrepressive activity of PEA3. Human breast cancer cells, MDA MB453, were cotransfected with 0.5 μg pNeulit along with increasing amounts of PEA3 plasmid DNA. The luciferase activity of each transfectant was measured. The doses of PEA3 from bar 1-6 are 0, 0.05, 0.5, 1, 2, 5 μg.*

A.


HER-2/neu-overexpressing cells
Basal HER-2/neu expressing cells
MDA MB453
SK-OV-3.ip1
MDA MB435
2774 c-10
PEA3
Vector control

B.

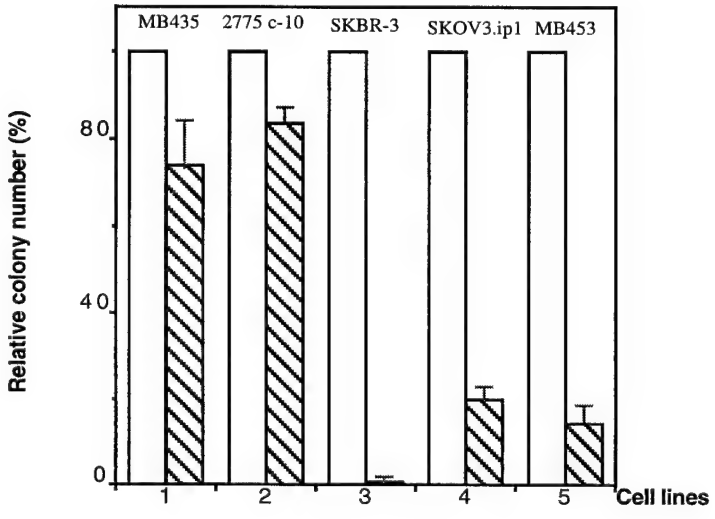

MB435
2775 c-10
SKBR-3
SKOV3.ip1
MB453
Relative colony number (%)
80
40
0
1
2
3
4
5
Cell lines

Figure 7 *PEA3 inhibits colony formation of HER-2/neu-overexpressing breast and ovarian cancer cells*
A. *Human breast and ovarian cells were transfected by 5 μg of either pCDNA3-PEA3 plasmid or vector control pCDNA3. Three days later, cells were split to 4 plates and cultured in 10% FCS DMEM in the presence of 500 μg/ml of G418 for 3 weeks. Colonies were stained with crystal violet and counted.* ***B.*** *Dramatic growth inhibition by PEA3 can be observed for HER-2/neu-overexpressing cell lines, which have less than 5% colonies from PEA3 transfection relative to that of the vector control. No significant change of colony number for the low expressors between vector control pCDNA3 and pCDNA3-PEA3 transfection.*

Table I *PEA3 suppresses the growth of cancer cells that overexpress HER-2/neu. Experiments were performed by transfecting equal amounts of PEA3 and pCDNA3 genes into cells and selecting the colonies in G418-supplement medium. Transfections in each cell lines were independently performed 2-4 times, using different batches of cells and DNA.*

	plasmid	
cell line	pCDNA3	PEA3
HER-2/neu overexpresser		
MDA-MB 453	100	19.7(±3.0)
SK-BR-3	100	0.7 (+0.9)
SKOV-3	100	14.3(+4.1)
HER-2/neu basal expresser		
MDA-MB 435	100	73.4(+10.9)
2774 c-10	100	83.3(+3.8)

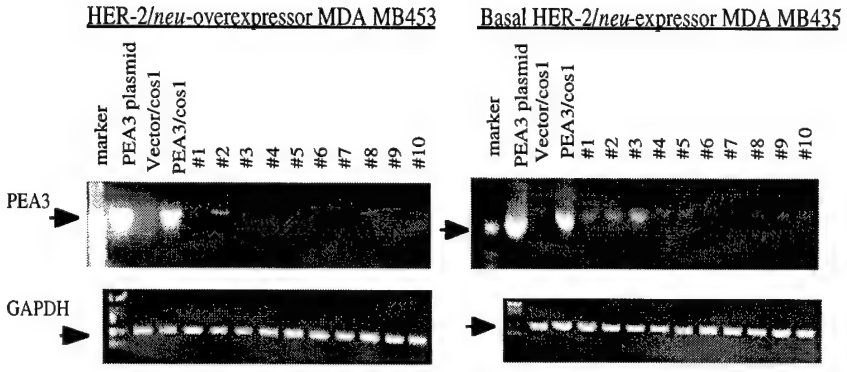


Figure 8 *Detection of PEA3 expression in transfecting colonies. Ten PEA3 transfected colonies from MDA MB453 and MDA MB435 respectively were examined for expression of PEA3 by RT-PCR using transgene PEA3 specific primers. Only 2 out of 10 MDA MB453 colonies have detectable PEA3 RNA while 10 out of 10 MDA MB435 colonies express PEA3 RNA. Primers of GAPDH were used as internal control. Arrows indicate the PCR products.*

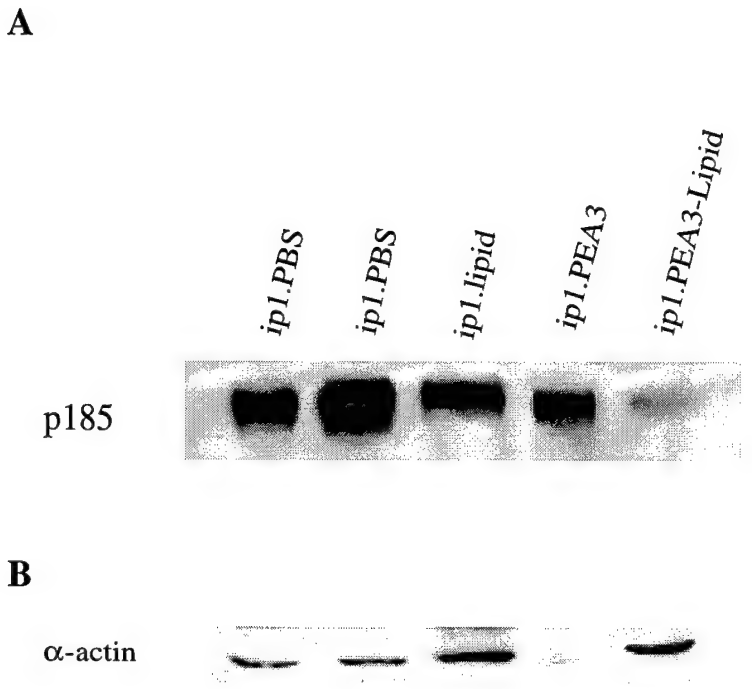
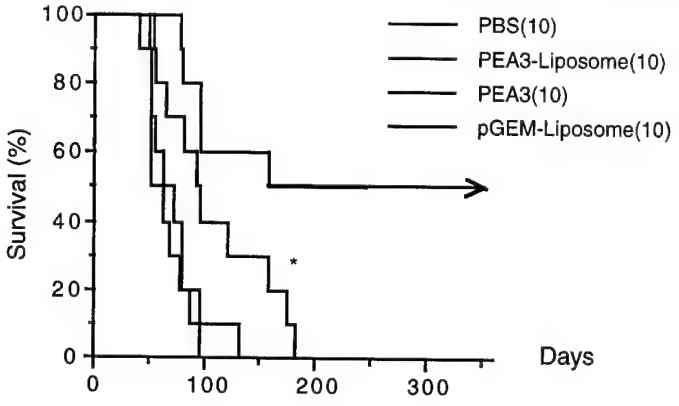


Figure 9 Immunoblot analysis of HER-2/neu-encoded p185 proteins in tumor lysates from mice of each indicated group. 50 μg of proteins from each sample were subjected to electrophoresis on 8% sodium dodecyl sulfate-polyacrymide gel prior to transfer to nitrocellulose filters. Filters were incubated with the primary antibody c-neu-Ab-3 against p185. Th eposition of the p185 proteins is shown to the left(A), the same filter was also incubated with the antibody against α-action, the signals indicate the protein loading(B).

A

SKOV3.ip1, human ovarian cancer cells that overexpress HER-2/neu



B

2774 c-10, human ovarian cancer cells with basal level of HER-2/neu expression

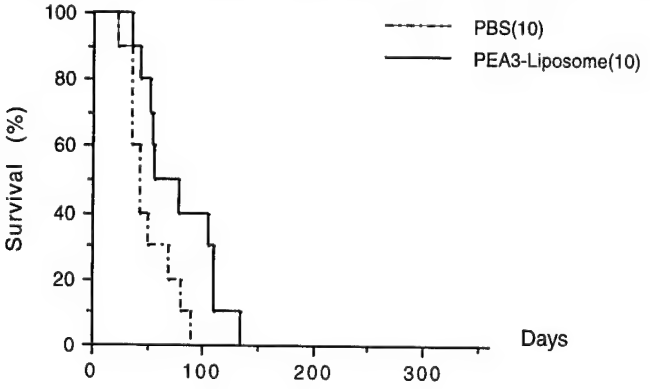


Figure 10 *ovarian cancer.* ***A****, Female nu/nu mice were induced tumors by injecting i.p. 2X10^6 of SKOV-3.ip1 cells 5 days before treatment. The mice received weekly i.p. injections of 200 μl of a reagent containing 15 μg of PEA3 DNA complex with 200 nmol of liposome, 15 μg of control DNA (pGEM) complex with 200 nmol of liposome, 15 μg of PEA3 DNA, 200 nmol of liposome, or PBS. There were 10 mice in each group. The survival of mice in each treatment group is expressed as a percentage of investigated animals (100%). * marks the last injection.* ***B****, 2774 c-10 cells with a basal level of HER-2/neu were inoculated i.p. and treated with PEA3-liposome complex by the same procedure as for SKOV-3.ip1 cells. There were 10 mice in each group.*

Discussion and Conclusions

Discussion

PEA3 belongs to the *Ets* family, which currently has 35 family members(30, 31). They share a highly homologous DNA-binding domain, yet have non-conserved trans-activation domains. There are 6 members of the *Ets* family gene are shown to encode sequence-specific DNA-binding proteins. Each of these proteins recognized similar motifs in the regulatory region of growth related genes, that share a centrally located 5'-GGAA-3' element. Although each of the *Ets* proteins known to bind to DNA that appears to recognize a common core sequence, data suggest that these proteins may not bind to identical sequences *in vitro*. Research data suggest that all of the *Ets* proteins recognize the same central core sequence but that each protein interacts with unique bases that flank this core(32, 33). The fact that expression of these *Ets* members being regulated differently through transcription or post-transcriptional modification also suggests a coordinated regulatory function of these *ets* genes. We propose that the trans-activation of *neu* expression through the PEA3 motif might be mediated by an *Ets* factor which contributes positively to cell transformation. PEA3 may compete for the DNA-binding site of *HER-2/neu* promoter and mitigate trans-activation of the gene. Scott et al showed that the promoter of the *HER-2/neu* gene contains an *ets*-binding site that is active in breast-cancer cells lines, and demonstrated the presence of an approximately 60-KDa *Ets* protein interacting with this *Ets*-binding site(34). This factor might be ERM, the member of PEA3 subfamily. This observation supports the model we proposed for the mechanism of PEA3 mediated transcriptional regulation of *HER-2/neu*. Another *Ets* family gene, ERF, has also been shown to repress transcription of the promoters that contain the *ets*-binding site, antagonize the trans-activation of other *ets* genes, and consequently suppress *Ets*-dependent transformation activity (35). It is worth to mention that in the *HER-2/neu* overexpressing human cancer cells, this competition may not exist since PEA3 does not express (such as in MDA MB453 and MDA MB134-VI,or ZR75-1) or the competition is partial due to the low level expression

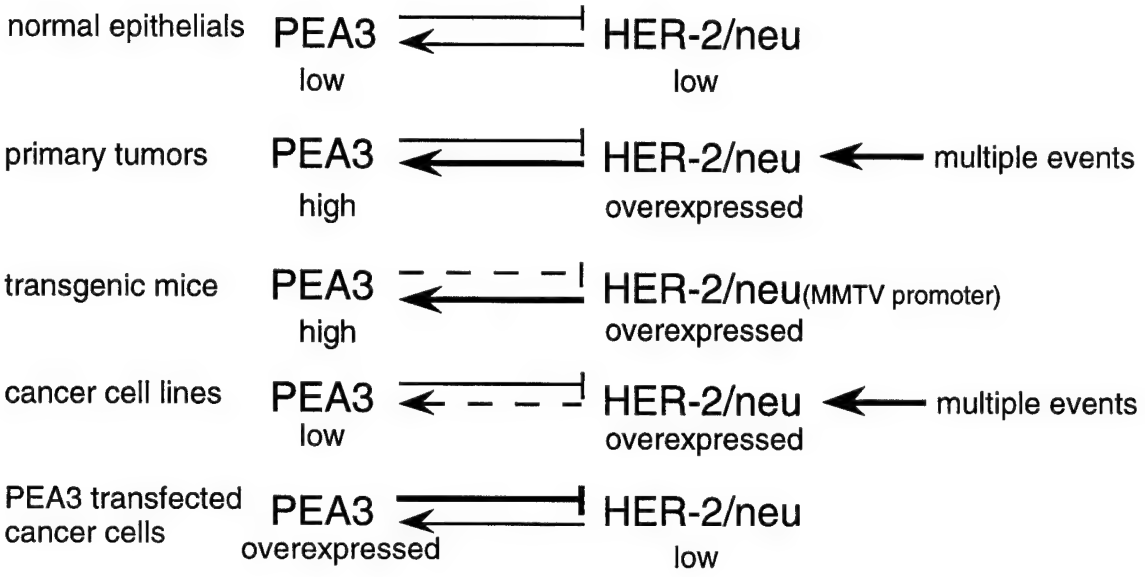
of PEA3(SKBR-3, BT474, MDA MB361, or BT483), therefore the *HER-2/neu* gene in still overexpressed as detected.

In a transgenic mouse study, a higher expression level of PEA3 mRNA was detected in the metastatic tumor nodules obtained from MMTV promoter-driven *neu*-transgenic mice. It suggests that *HER-2/neu* upregulates the PEA3 gene expression. A transfection experiment also shown that the transiently expression of *HER-2/neu* in COS1 cells resulted in the higher activity of PEA3 promoter in a luciferase reporter construct (36). However, studies on the human breast cancer cell lines detected a consistently low expression of PEA3 in the *HER-2/neu*-overexpressing cells compared to the normal epithelial breast cells, strongly suggesting that the expression of PEA3 might be required for a normal level expression of *HER-2/neu.* A possible explanation for all these data could be: In the normal cells, PEA3 is a trans-repressor and an *Ets* family member (i.e ERM) is a trans-activator for the *HER-2/neu* transcription, both mediated their effects through one specific DNA element, the PEA3 binding site. The coordinate activity of PEA3 and the *Ets* factor contributes to the maintenance of the basal level *HER-2/neu* proto oncogene. Expression of *HER-2/neu* also upregulates PEA3 transcription, these two molecules thus form a feedback regulation loop. PEA3 is thus a more critical controller for *HER-2/neu* gene expression. In the breast tumor, an unknown initiating event results in enhanced *HER-2/neu* gene expression and the PEA3 expression was thus increased by *HER-2/neu.* The high expression of PEA3 somehow is not sufficient to mediate the downregulation of *HER-2/neu* transcription. In human cancer cell line, the signal pathway leads to the up-regulation of PEA3 by *HER-2/neu* is somehow abolished. The absence of negative regulator PEA3 on the *HER-2/neu* transcription helps this gene to be overexpressed. Thus, a low or no PEA3 expression is observed in the *HER-2/neu* overexpressing cancer cells. In the transgenic mice system, the constitutive expression of *HER-2/neu* resulted in the up-regulation of PEA3, and PEA3 was detected as overexpressed in these cells. The expression of *HER-2/neu* in cells of transgenic mice up-regulated the PEA3 level, however PEA3 is unable to repress *neu* transcription in this scenario, since *HER-2/neu* transcription is under the control of MMTV promoter not its own promoter. Thus the feedback loop is blocked

and the both genes are highly expressed in a same cell(Model 1). Benz *et al* reported recently that PEA3 transactivated the *HER-2/neu* gene expression in COS-1 cells with transient co-transfection experiments(36). It seems to be an interesting observation since side-by-side experiments showed that PEA3 transrepressed rather than transactivated *HER-2/neu* promoter in the ovarian and breast cancer cell lines we tested. The biological significance of the *HER-2/neu* transcriptional up-regulation by PEA3 in COS1 cells remains to be determined.

It has been reported that 85% of breast cancer cells express low levels of PEA3 and that the *HER-2/neu* overexpressing breast cancer cells express no or very little PEA3(24). In this study, we have shown that ectopic expression of PEA3 gene selectively inhibits the growth of cancer cells that overexpress *HER-2/neu*. In a model system, the expression of genomic *HER-2/neu* in mouse fibroblast NIH 3T3 cells induces cellular transformation envisioned by formation of foci, which is suppressed by co-expression of PEA3 in these cells. These results strongly suggest that PEA3 exhibits a strong suppression activity on *HER-2/neu*-mediated transformation, and that the PEA3 may potential function as a tumor suppressor gene in the *HER-2/neu*-overexpressing human cancers (Proposed Tasks 1 and 2).

The abnormal regulation of gene expression and the consequent aberrant cell proliferation is one cause of cellular transformation; *Ets* family transcriptional factors are believed to play a role in cell growth control and transformation. *Ets1* p51 has been shown to reverse the transformation phenotype in colon cancer cells, and its derivative, p42, suppresses transformation of colon cancer cells through induction of apoptosis(35, 36). Expression of *Ets2* reverse the transformation phenotype-mediated by the oncogenic *ras*. The expression of a tumor suppression gene maspin is lost in high malignant cancer cells and this loss results at least in part from the absence of trans-activation activity through PEA3 and Ap1 elements in its promoter(23). PEA3 and AP1 regulatory activity favors the tumor suppression mechanism. These data strongly support the concept that *Ets* family genes maybe critical to normal cell physiology and may exhibit tumor-suppression activity in cancer cells. PEA3 has also been shown to upregulate the transcriptional activity of several protease genes



Model 1 *Model for regulation loop of PEA3 and HER-2/neu expression in normal cancer and neu-transgenic cells.*

(39, 40, 41). PEA3 thus is also an important regulator for cancer cell metastasis. There is no direct evidence yet showing that PEA3 gene expression increases cell mobility or induces a metastatic phenotype.

The best way to explore the biological consequence of PEA3 expression is to set up a cancer cell line that ectopically expresses PEA3. Due to the growth inhibition effects of PEA3 expression, such cells would be established in the tetracyclin-inducible gene expression system, and the PEA3 gene expression would be controlled by the addition of tetracyclin or its derivative doxycyclin. These cell lines will allow us to systematically examine the biological effects of PEA3 on *HER-2/neu*-overexpressing cancer cells, specifically on cell growth rate and the transformation phenotypes such as anchorage dependent and independent growth, tumorigenicity in mammary fat pad in mouse. The metastatic phenotypes of these cells will also be tested *in vitro* and *in vivo*. We would also like to investigate the molecular events driven these biological changes. We believe that these studies will help us to further assess transcriptional regulatory function of PEA3 in the normal mammary cells and unveil its role in cell motility and transformation(Proposed Tasks 1 and 2).

The expression of PEA3 gene is capable of inhibiting the growth of cancer cells and suppressing the *HER-2/neu*-mediated transformation. Though we have not finished the task of examining whether PEA3 expression in Adenovirus vector exhibits the anti-tumor activity in breast cancer (Proposed Task 3), our experimental result using PEA3-liposome mediated gene transfer strongly support that PEA3 is associated with an anti-tumor activity and may be potentially used as a therapeutic gene for *HER-2/neu*-overexpressing human cancers *in vivo* (Proposed Task 2). We are aware that PEA3 is a transcriptional factor, the PEA3 motif exists on the promoters of many cellular genes. Reintroduction of PEA3 into cancer cells *in vivo* affects a set of other genes expression besides *HER-2/neu*, and may potentially induce side effects. For example the upregulation of proteinase genes may increase cancer cell mobility and invade to the surrounding tissue. The transcriptional regulation of these gene by PEA3 may require its transcriptional regulation domain

as well as the DNA binding domain. The PEA3-DBD, which is sufficient to repress *HER-2/neu* promoter, will significantly reduce the side effects mediated the PEA3 transcriptional regulation domain, and may be a better candidate than the wild-type PEA3 for a gene therapy setting in the future.

Conclusions

1. PEA3 binds to the *HER-2/neu* promoter specifically through PEA3 motif.
2. PEA3 represses *HER-2/neu* transcription in human breast and ovarian cancer cells.
3. PEA3 suppresses the activated *neu*-mediated transformation in focus forming assay.
4. PEA3 exhibits transcriptional repression of *HER-2/neu* gene by competition with a transcriptional activator in binding to the *HER-2/neu* promoter in human cancer cells.
5. The DNA binding domain of PEA3 is sufficient for its transrepressive activity.
6. PEA3 selectively inhibits the colony formation of *HER-2/neu*-overexpressing breast and ovarian cancer cells.
7. PEA3-liposome preferentially suppresses the development of cancer cells in the orthotopic cancer model, the effects are likely mediated through downregulating the expression of *HER-2/neu* protein.

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EDUCATION/TRAINING

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RESEARCH AND PROFESSIONAL EXPERIENCE:

Honors/Awards:

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1997 Presidential Fellowship-Special Commendation Award, UT-Houston GSBS
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Research experience after degree of bachelor in science:

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Publications:

1. **Xing, X**. The Tertiary Structure of Glucose Isomerase.(1990) Thesis for B.S.
2. **Xing, X**. The Effects of Temperature Sensitive Liposome on Targeting and Selectively Drug-Releasing to Liver Cancer Cells. (1993) Thesis for M.S.
3. Hung, M-C., Matin, A., Zhang, Y., **Xing, X**., Sorgi, F., Huang, L., and Yu, D. (1995) *HER-2/neu* targeting gene therapy-a review. *Gene*, 159:65-71.
4. **Xing, X.,** Matin, A., Yu, D., Xia, W., Sorgi, F., Huang, L., and Hung, M-C. Mutant SV40 large T as a therapeutic agent for *HER-2/neu*-overexpressing ovarian cancer. *Cancer Gene Therapy* 3:168-174, 1996.
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Abstracts:

1. **Xing, X.** and Hung M.-C. Tumor Suppression by Large T antigen in *HER-2/neu* overexpressing human ovarian cancer cells.
   Cold Spring Harbor 1996 meeting on GENE THERAPY, Cold Spring Harbor, 1996.

2. **Xing, X.,** Matin, A., Yu, D., Xia, W., and Hung, M.-C. Mutant SV40 large T as a therapeutic agent for *HER-2/neu*-overexpressing ovarian cancer.
   9th International Congress On Breast Disease of Senologic International Society, Houston, 1996.
   And *Regulatory Mechanisms in Growth and Differentiation*-49th Annual Symposium on Fundamental Cancer Research, Houston, 1996.

3. Miller, S. J., **Xing, X.,** Xi, L. and Hung, M.-C. Identification of a specific DNA region requiredfor enhanced transcription of *HER-2/neu* in the MDA MB453 breast cancer cell line.
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4. **Xing, X.,** Miller SJ. and Hung M.-C. PEA3 serves as a therapeutic gene in human cancer cells through downregulation of HER-2/*neu*.
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   And *Trainee Recognition Day & Research Exposition*, U.T. M.D.Anderson Associates, 1997.